REPORT

EVALUATION OF THE MUTAGENIC ACTIVITY OF

[redacted]

IN AN *IN VITRO* MAMMALIAN CELL GENE MUTATION TEST

WITH L5178Y MOUSE LYMPHOMA CELLS

(WITH INDEPENDENT REPEAT)

NOTOX Project 346332
NOTOX Substance 111834/B

CONFIDENTIALITY STATEMENT

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STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice

which are essentially in conformity with:

The United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director

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QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was audited by the NOTOX Quality Assurance Unit to ensure that the methods and results accurately reflect the raw data.

The dates of Quality Assurance inspections and audits are given below.
During the on-site inspections procedures applicable to this type of study were inspected.

DATES OF QAU INSPECTIONS/ AUDITS	REPORTING DATES
on-site inspection	
18-04-2002 to 24-04-2002 (process)	08-05-2002
protocol inspection	
28-02-2002	28-02-2002
report audit	
12-06-2002	12-06-2002

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Date: July 01, 2002

SUMMARY

This report describes the effects of [redacted] on the induction of forward mutations at the thymidine-kinase locus (TK-locus) in L5178Y mouse lymphoma cells in the presence and absence of S9-mix. The test was performed in two independent experiments in the presence and absence of S9-mix (Aroclor-1254 induced rat liver S9-mix).

A range finding study was performed to set dose levels for the subsequent mutation studies and to establish the solubility of [redacted]. [redacted] precipitated in the exposure medium at a test substance concentration of 325 µg/ml.

In the first experiment, [redacted] was tested up to concentrations of 90 and 300 µg/ml in the absence and presence of 8 % (v/v) S9-mix, respectively. Incubation time was 3 hours. Appropriate toxicity was observed at these dose levels in the absence and presence of S9-mix.

In the second experiment, [redacted] was tested up to concentrations of 225 and 350 µg/ml in the absence and presence of 12 % (v/v) S9-mix, respectively. Incubation times were 24 hours and 3 hours for incubations in the absence and presence of S9 metabolic activation respectively. Toxicity was observed at the dose level of 225 µg/ml in the absence of S9-mix. In the presence of S9-mix, [redacted] was tested beyond the limit of the solubility and showed no toxicity at this dose level.

Mutant frequencies in cultures treated with positive control chemicals were increased by 11- and 40-fold for EMS in the first and second experiment respectively, and by 13- and 10-fold for DMN, in the first and second experiment respectively. It was therefore concluded that the test conditions, both in the absence and presence of S9-mix, were appropriate for the detection of a mutagenic response and that the metabolic activation system (S9-mix) functioned properly.

[redacted] did not induce a significant increase in the mutant frequency in the absence or presence of S9 metabolic activation in the first experiment. This result was confirmed in a second, repeat experiment with modifications in the duration of treatment in the absence of S9, and in the composition of the S9 concentration for metabolic activation.

It is concluded that [redacted] is not mutagenic in the mouse lymphoma L5178Y test system under the experimental conditions described in this report.

PREFACE

Sponsor	[redacted]
Study Monitor	Dr. C.L.J. Braun SHERA, Regulatory Affairs
Testing Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	C.M. Verspeek-Rip
Technical Coordinator	N.P. Giepmans
Study Plan	Start : 18 March 2002 Completed : 22 April 2002

TEST SUBSTANCE

Identification Chemical name CAS RN	[redacted]
Description	Clear colourless liquid
Batch	1510-14
Purity	See Certificate of Analysis (Appendix 2)
Test substance storage	In refrigerator in the dark
Stability under storage conditions	Stable
Expiry date	01 January 2003
Density	Approx. 1160 $kg.m^{-3}$
Stability in vehicle	Dimethyl sulfoxide: Unknown

The sponsor is responsible for all test substance data unless determined by NOTOX.

Note: **Don't heat up the test substance above 50°C**

ARCHIVING

NOTOX B.V. will archive the protocol, report, test article reference sample and raw data for at least 10 years. No data will be withdrawn without the sponsor's written consent.

GUIDELINES

The study procedures described in this report were based on the following guidelines:
- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Guideline no. 476: "Genetic Toxicology: In Vitro Mammalian Cell Gene Mutation Tests", (adopted July 21, 1997).
- European Economic Community (EEC). Adapting to technical progress for the twenty-sixth time Annex V of the EEC Directive 67/548/EEC, Part B: Methods for the Determination of Toxicity; B.17: "Mutagenicity: "In vitro mammalian cell gene mutation test". EEC Publication Commission Directive (Published June 8, 2000).

OBJECTIVE

Objective

The objective of this study was to evaluate the mutagenic potency of [redacted] by testing its ability to induce forward mutations at the thymidine kinase (TK) locus in L5178Y mouse lymphoma cells, either in the absence or presence of a metabolic system (S9-mix).

Background of the test system

L5178Y mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes. The TK mutational system is able to detect base pair alterations, frame shift mutations and small deletions.
Cells deficient in thymidine kinase (TK), due to the forward mutation ($TK^{+/-}$ to $TK^{-/-}$) are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). TK deficient cells can not incorporate the analogue into its phosphorylated derivative (nucleotide); the nucleotides needed for cellular metabolism are obtained solely from *de novo* synthesis. In the presence of TK, TFT is converted into nucleotides, which are lethal to the cells. Thus, cells, which will survive in culture medium containing TFT, are mutated, either spontaneously or by the action of the test substance, giving rise to a TK deficient phenotype. Furthermore, by applying the TFT-selection procedure it is possible to discriminate between the two different classes of TFT-resistant mutants (small and large colonies) which are believed to represent the different types of lesions induced in the DNA by the test substance.
A test article that induces a positive response in this assay is presumed to be a potential mammalian cell mutagen.

MATERIALS AND METHODS

TEST SYSTEM

Test System	L5178Y mouse lymphoma cells
Rationale	Recommended test system in international guidelines (e.g. EPA, OECD, EEC).
Source	Dr. A.G.A.C. Knaap, Department of Radiation Genetics and Chemical Mutagenesis of the State University of Leiden, The Netherlands (1981). This mouse lymphoma cell line was originally derived from the Fischer L5178Y line, isolated by Clive (1975).

Stock cultures of these cells were stored in liquid nitrogen (-196°C). The cultures were checked for mycoplasma contamination.

CELL CULTURE

F10 complete culture medium	F10 complete culture medium consisted of Ham's F10 medium without thymidine and hypoxanthine (Gibco), supplemented with 10% (v/v) horse serum, L-glutamine (2 mM) and penicillin/streptomycin (50 U/ml and 50 µg/ml respectively).
Cell culture conditions	L5178Y mouse lymphoma cells were cultured in F10 complete culture medium. Cell density was preferably kept below 7 x 10^5 cells/ml.
Exposure medium	Cells were exposed to [redacted] for 3 hours in F10 culture medium buffered with 20 mM HEPES or for 24 hours in F10 complete culture medium.
Selective medium	Selective medium consisted of F10 complete culture medium, supplemented with 10% (v/v) horse serum and 5 µg/ml TFT (Sigma).
Non-selective medium	Non-selective medium consisted of F10 complete culture medium, supplemented with 10% horse serum.
Environmental conditions	All incubations were carried out in a humid atmosphere (80-100%) containing 5 ± 0.5 %CO_2 in air in the dark at 37 ± 1 °C. The temperature and CO_2-percentage were monitored during the experiment.

TREATMENT OF THE TEST SUBSTANCE

The test substance was dissolved in dimethyl sulfoxide of spectroscopic quality (Merck). Test substance concentrations were prepared directly prior to use. The final concentration of the solvent in the exposure medium was 0.8% (v/v).

REFERENCE SUBSTANCES

Negative control:

The solvent for the test article, i.e. dimethyl sulfoxide.

Positive controls:

Solvents for Reference Substances
Hanks' balanced salt solution without calcium and magnesium.

Solutions of reference substances were prepared immediately before use.

Without metabolic activation (-S9-mix):
Ethylmethanesulphonate (EMS; CAS no. 62-50-0; purity 98%; Janssen Chimica) was used as a direct acting mutagen at a final concentration of 2 mM for a 3 h treatment period and 1 mM for a 24 h treatment period.

With metabolic activation (+S9-mix):
Dimethylnitrosamine (DMN; CAS-no. 62-75-9, purity 99+%, Acros) (0.5 mM) was used. DMN had to be activated by microsomal enzymes present in the S9-mix, resulting in a methyldiazonium ion which could react with cellular DNA.

METABOLIC ACTIVATION SYSTEM

Preparation of S9-fraction

Rat liver microsomal enzymes were routinely prepared from adult male Wistar rats, which were obtained from Charles River, Sulzfeld, Germany.

The animals were housed at NOTOX in a special room under standard laboratory conditions, as described in the Standard Operating Procedures. The rats were injected intraperitoneally with a solution (20% (w/v)) of Aroclor 1254 (500 mg/kg body weight) in corn oil. Five days later, they were killed by decapitation; (they were denied access to food for at least 12 hours preceding sacrifice).
The livers of the rats were removed aseptically, and washed in cold (0°C) sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Na_2-EDTA. Subsequently the livers were minced in a blender and homogenized in 3 volumes of phosphate buffer with a Potter homogenizer. The homogenate was centrifuged for 15 min at 9000 g. The supernatant (S9-fraction) was transferred into sterile ampules, which were stored in liquid nitrogen (-196°C).

Preparation of S9-mix

S9-mix was prepared immediately before use and kept on ice. S9-mix contained per ml: 1.63 mg $MgCl_2.6H_2O$; 2.46 mg KCl; 1.7 mg glucose-6-phosphate; 3.4 mg NADP and 4 µmol HEPES. The above solution was filter (0.22 µm)-sterilized. To 0.5 ml S9-mix components 0.5 ml S9-fraction was added (50% (v/v) S9-fraction) to complete the S9-mix in the first experiment and to 0.3 ml S9-mix components 0.7 ml S9-fraction was added (70% (v/v) S9-fraction) to complete the S9-mix in the second experiment.

Metabolic activation was achieved by adding 0.2 ml S9-mix to each ml cell suspension. The concentration of the S9-fraction in the exposure medium was 8% (v/v) in the dose range finding test and the first experiment and 12% (v/v) in the second experiment. The S9-batches used were 02-1 and 02-2.

EXPERIMENTAL PROCEDURE

Cleansing

Prior to dose range finding and mutagenicity testing, the mouse lymphoma cells were grown for 1 day in F10 complete culture medium containing 10^{-4} M hypoxanthine, 2×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine (HAT-medium) to reduce the amount of spontaneous mutants, followed by a recovery period of 2 days on medium containing hypoxanthine and thymidine only. After this period cells were returned to normal medium at least for 1 day before starting the experiment.

Dose range finding test

In order to select appropriate dose levels for mutagenicity testing, cytotoxicity data were obtained by treating 8×10^6 cells, suspended in 8 ml exposure medium with a number of test substance concentrations increasing with approximately half log steps. The cell cultures were treated in sterile 30 ml centrifuge tubes.

Since [redacted] was poorly soluble in aqueous solution, the highest tested concentration was 333 µg/ml exposure medium.

Cell cultures were exposed to [redacted] in exposure medium for 3 hours in the presence of S9-mix and for 3 and 24 hours in the absence of S9-mix. The cells were treated in a shaking incubator at 37 ± 1 °C and 145 spm for 3 and 24 hours. After exposure, the cells were separated from treatment solutions by 2 centrifugation steps (115 g, 8 min) each followed by removal of the supernatant. The first centrifugation step was followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the final centrifugation step the cells were resuspended in F-10 complete culture medium. The cells in the final suspension were counted with a microscope with an "Improved Neubauer" haemocytometer.

For determination of the cytotoxicity, the surviving cells of the 3 hours treatment were subcultured twice. After 24 hours of subculturing, the cells were counted and subcultured again for another 24 hours, after that the cells were counted. The surviving cells of the 24 hours treatment were subcultured once. After 24 hours of subculturing, the cells were counted. If less than 1.6×10^5 cells were counted no subculture was performed.

The relative cytotoxicity expressed as the reduction in cell growth after approximately 24 and 48 hrs or only 24 hours cell growth, compared to the cell growth of the solvent control, was used to determine an appropriate dose range for the mutagenicity tests.

Mutagenicity test

[redacted] was tested both in the absence and in the presence of S9-mix in two independent experiments. Per culture 8 x 10^6 cells (10^6/ml) were used. If test substance concentrations were expected to be toxic, 16 x 10^6 cells (10^6/ml) were used per culture.

The concentration of the S9-fraction in the exposure medium was 8% (v/v) and 12% (v/v) in the first and second experiment respectively.

In the first experiment, cell cultures were exposed for 3 hours to [redacted] in exposure medium. In the second experiment, cell cultures were exposed to [redacted] in exposure medium for 24 hours in the absence of S9-mix and for 3 hours in the presence of S9-mix. The cell cultures were treated in centrifuge tubes in a shaking incubator at 37 ± 1 °C and 145 spm. Solvent and positive controls were included. The solvent control was tested in duplicate.

After exposure, the cells were separated from treatment solutions by 2 centrifugation steps (115 g, 8 min) each followed by removal of the supernatant. The first centrifugation step was followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the final centrifugation step the cells were resuspended in F-10 complete culture medium. The cells in the final suspension were counted with a microscope with an "Improved Neubauer" haemocytometer.

Cloning efficiency directly after treatment

Immediately after exposure to [redacted] 2 x 96-well microtiter plates were set up for each dose with 1 cell/well, to determine the survival of day 0. The plates were scored after 9 days incubation for determination of the cloning efficiency.

Expression period

For expression of the mutant phenotype, the remaining cells of the cell cultures treated for 24 hours with various doses of [redacted] were cultured for 2 days. The remaining cells of the cell cultures treated for 3 hours with various doses [redacted] were cultured for 3 days. During this culture period at least 4 x 10^6 cells (if possible) were subcultured at least every other day in order to maintain log phase growth. Three days after the start of the treatment with the test substance the cells were plated for determination of the cloning efficiency (CE_3) and the mutant frequency (MF).

Determination of the mutant frequency

Eight doses of the test substance were selected for the mutation assay, both in the absence and presence of S9-mix.

For determination of the CE_3 the cell suspensions were diluted and seeded in wells of a 96-well dish. 1 cell was added per well (2 x 96-well microtiter plates/concentration) in non selective medium.

For determination of the MF a total number of 9.6 x 10^5 cells/concentration were plated in four 96-well microtiter plates, each well containing 2500 cells in selective medium (TFT-selection). The microtiter plates for CE_3 and MF were incubated at 37°C in humidified air with 5% CO_2 for 10 or 11 days.

After this incubation period the plates for the TFT-selection were stained for 2 hours, by adding 0.5 mg/ml MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) to each well. The plates for the CE_3 and MF were scored with the naked eye or with the microscope.

The calculation of the cloning efficiency was determined by dividing the number of empty wells by the total number of wells. This value was called P(0), the zero term of the Poisson distribution.
P(0) = number of empty wells/total number of wells
CE = -ln P(0)/number of cells plated per well

The calculation of the mutation frequency was determined, as follows:
MF = {-ln P(0)/number of cells plated per well}/CE_3.
The mutant frequency was expressed as the number of mutants per 10^5 surviving cells.

DETERMINATION OF THE MUTANT COLONIES

The colonies were divided into small and large colonies. Mutant cells that have suffered extensive genetic damage have prolonged doubling times and thus form small colonies. Less severe affected mutant cells have grown at rates similar to the parental cells and form large colonies. The small colonies can be associated with the induction of chromosomal mutations. The large colonies appeared to result from mutants with single gene mutations (substitutions, deletions of base-pairs) affecting the TK gene.

The small colonies are morphological dense colonies with a sharp contour and with a diameter less than a quarter of a well. The large colonies are morphological dense colonies with a hazy contour and with a diameter larger than a quarter of a well.

ACCEPTABILITY OF ASSAY

A mutation assay was considered acceptable if it met the following criteria:
a) The absolute cloning efficiency of the solvent controls was $\geq$ 50%.
b) In at least seven of the eight doses of the test substance, an acceptable number of surviving cells (10^6) could be analysed for expression of the TK mutation.
c) The spontaneous mutant frequency in the untreated or solvent control was < 10 per 10^5 clonable cells.
d) The positive controls (ethylmethanesulfonate and dimethylnitrosamine) induced significant (at least three-fold) increases in the mutant frequencies.

DATA EVALUATION AND STATISTICAL PROCEDURES

The experimental results were not subjected to statistical analysis.
A test substance was considered positive (mutagenic) in the mutation assay if:
a) It induced at least a 3-fold increase in the mutant frequency compared to the solvent control in a dose-dependent manner; and
b) The results were reproducible in an independently repeated test.

A test substance was considered negative (not mutagenic) in the mutation assay if:
a) None of the tested concentrations showed a mutant frequency of at least three-fold compared to the solvent control.
b) The results were confirmed in an independently repeated test.

RESULTS

DOSE RANGE FINDING TEST

Solubility

[redacted] precipitated in the exposure medium at a concentration of 333 µg/ml. Therefore, this concentration was used as the highest test substance concentration in the dose range finding study.

Dose range finding test

In the dose range finding test, L5178Y mouse lymphoma cells were treated with a test substance concentration range of 1 to 333 µg/ml in the absence of S9-mix with a 3 and 24 hour treatment period and in the presence of S9-mix with a 3 hour treatment period.

Table 1 shows the cell counts of the cultures after 3 hours of treatment with various concentrations of [redacted] and after 24 and 48 hours of subculture and the calculated suspension growth and the relative suspension growth.

In the absence of S9-mix after 3 hours of treatment, no toxicity in the suspension growth was observed up to concentrations of 33 µg/ml compared to the suspension growth of the solvent control. No cell survival was observed at test substance concentrations of 100 and 333 µg/ml after 3 hours treatment.

In the presence of S9-mix after 3 hours of treatment, no toxicity in the suspension growth was observed up to concentrations of 100 µg/ml compared to the suspension growth of the solvent control. No cell survival was observed at the test substance concentration of 333 µg/ml after 3 hours treatment and 24 hours of subculture.

Table 2 shows the cell counts of the cultures after 24 hours of treatment with various concentrations of [redacted] and after 24 hours of subculture and the calculated suspension growth and the relative suspension growth.

In the absence of S9-mix after 24 hours of treatment, no toxicity in the suspension growth was observed up to concentrations of 100 µg/ml compared to the suspension growth of the solvent control. No cell survival was observed at the test substance concentration of 333 µg/ml after 24 hours of treatment.

MUTAGENICITY TEST

Tables 3 and 4 show the percentages of cell survival and the mutant frequencies for various concentrations of TRIGONOX R-938. Individual colony counts of cloning and selective plates, and cell counts during subculturing are listed in Tables 5-12 of Appendix 1.

Experiment 1

Based on the results of the dose range finding test and the solubility test, the following dose range was selected for the first mutagenicity test:

Without S9-mix: 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 µg/ml exposure medium.
With 8% (v/v) S9-mix: 1, 10, 30, 50, 70, 100, 130, 175, 230, 275 and 300 µg/ml exposure medium.

Evaluation of toxicity
In the absence of S9 metabolic activation, the dose levels of 0.5 up to and including 50 µg/ml showed no severe difference in the cell growth. Therefore the dose levels of 0.5, 1 and 5 and 10 µg/ml were not regarded relevant for mutant frequency measurement. In the presence of S9 metabolic activation, the dose levels of 1 up to and including 175 µg/ml showed no severe difference in the cell growth. Therefore the dose levels of 1, 30 and 70 µg/ml were not regarded relevant for mutant frequency measurement.
The dose levels selected to measure mutant frequencies at the TK-locus were:
Without S9-mix: 20, 30, 40, 50, 60, 70, 80 and 90 µg/ml exposure medium.
With S9-mix: 10, 50, 100, 130, 175, 230, 275 and 300 µg/ml exposure medium.

In the absence of S9-mix (Table 3), after 3 hours treatment the actual cell survival of the highest test substance concentration was reduced by 78% compared to the actual cell survival of the solvent controls.

In the presence of S9-mix, after 3 hours treatment the actual cell survival of the highest test substance concentration was reduced by 65% compared to the actual cell survival of the solvent controls.

Evaluation of mutagenicity
[redacted] did not induce an increase in mutant frequency at the TK locus either in the absence or in the presence of S9-mix. The number of small and large colonies were comparable to the number of small and large colonies of the solvent controls.

Experiment 2
To obtain more information about the possible mutagenicity of [redacted] a second mutation experiment was performed. In the test without S9-mix the treatment period was extended to 24 hours. In the test with metabolic activation the amount of S9-mix was increased to 12% (v/v) and the treatment period remained at 3 hours.

Further investigation showed that at a concentration of 325 µg/ml [redacted] already precipitated in the exposure medium. [redacted] was tested beyond the limit of the solubility to obtain adequate mutagenicity data.

Based on the results of the dose range finding test and experiment 1, the following dose levels were selected for mutagenicity testing.
Without S9-mix: 10, 50, 75, 100, 130, 175, 200, 225, 250, 275, 300 and 325 µg/ml exposure medium.
With 12% (v/v) S9-mix: 10, 50, 100, 130, 175, 225, 250, 275, 300, 325 and 350 µg/ml exposure medium.

Evaluation of toxicity
In the absence of S9 metabolic activation, the dose levels of 250 µg/ml and upwards were not used for mutant frequency measurement, since this dose levels were too toxic for further testing. In the presence of S9 metabolic activation, no severe difference in the cell growth was observed in all dose levels tested and the dose levels of 10, 50 and 100 µg/ml were not regarded relevant for mutant frequency measurement.
The dose levels selected to measure mutant frequencies at the TK-locus were:
Without S9-mix: 10, 50, 75, 100, 130, 175, 200 and 225 µg/ml exposure medium.
With S9-mix: 130, 175, 225, 250, 275, 300, 325 and 350 µg/ml exposure medium.

In the absence of S9-mix (Table 4), after 24 hours treatment the actual cell survival of the highest test substance concentration of 225 µg/ml was reduced by 92% compared to the actual cell survival of the solvent controls.

In the presence of S9-mix, no severe toxicity was observed after 3 hours treatment.

Evaluation of mutagenicity
[redacted] did not induce the mutant frequency at TK locus either in the absence or in the presence of S9-mix.

DISCUSSION

The spontaneous mutant frequencies in the solvent-treated control cultures were between the minimum and maximum value of the historical control data range ({0.7- and 6.3 x 10^5 (mean 2.7 x 10^5) in the absence of S9-mix} and {0.7- and 6.9 x 10^5 (mean 3.1 x 10^5) in the presence of S9-mix}; for n=164 and 151 respectively).

Mutant frequencies in cultures treated with positive control chemicals were increased by 11- and 40-fold for EMS in the first and second experiment respectively, and by 13- and 10-fold for DMN, in the first and second experiment respectively (Table 3 and 4). It was therefore concluded that the test conditions, both in the absence and presence of S9-mix, were appropriate for the detection of a mutagenic response and that the metabolic activation system (S9-mix) functioned properly.

Mutant frequency at the TK-locus
As shown in tables 3 and 4, [redacted] did not induce a significant increase in the mutant frequency in the absence or presence of S9 metabolic activation in the first experiment. This result was confirmed in a second, repeat experiment with modifications in the duration of treatment in the absence of S9, and in the composition of the S9 concentration for metabolic activation.

CONCLUSION

In conclusion, [redacted] is not mutagenic in the TK mutation test system under the experimental conditions described in this report.

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TABLE 1 DOSE RANGE FINDING TEST OF [redacted] IN THE MOUSE LYMPHOMA L5178Y TEST SYSTEM

Dose range finding test (at 3 hours treatment)

Dose (µg/ml)	Cell count after 3 hours of treatment (cells/ml x 10^5)	Cell count after 24 hours of subculture (cells/ml x 10^5)	Cell count after 48 hours of subculture (cells/ml x 10^5)	Suspension growth Total [4]	Suspension growth % of control
		Without metabolic activation			
Solvent control	5.4	4.7	3.9	39	100
1	5.5	5.2	3.9	44	113
3	5.3	4.8	4.0	40	103
10	4.6	5.0	3.9	35	90
33	5.5	4.3	4.4	41	105
100	(2)	-	-	-	-
333 [1]	(2)	-	-	-	-
		With 8% (v/v) metabolic activation			
Solvent control	5.7	5.5	4.3	53	100
1	5.6	5.3	4.2	49	92
3	5.0	5.9	4.2	48	91
10	5.5	5.6	3.4	41	77
33	6.1	5.7	3.5	48	91
100	6.8	5.2	3.5	48	91
333 [1]	0.1 [3]	(2)	-	-	-

Solvent control = dimethyl sulfoxide

(1) [redacted] precipitated in the exposure medium

(2) Cell death

(3) No subculture

(4) $\text{Total growth} = \text{Cell count after 3 h treatment} \times \frac{\text{Cell count after 24 h subculture}}{\text{Cells subcultured after treatment } (1.6 \times 10^5 \text{ c/ml})} \times \frac{\text{Cell count after 48 h subculture}}{\text{Cells subcultured after 24 h } (1.6 \times 10^5 \text{ c/ml})}$

TABLE 2 DOSE RANGE FINDING TEST OF [redacted] IN THE MOUSE LYMPHOMA L5178Y TEST SYSTEM

Dose range finding test (at 24 hours treatment)

Dose (µg/ml)	Cell count after 24 hours of treatment (cells/ml x 10^5)	Cell count after 24 hours of subculture (cells/ml x 10^5)	Suspension growth Total (3)	Suspension growth % of control
		Without metabolic activation		
Solvent control	3.5	4.4	9.6	100
1	4.8	4.6	13.8	144
3	3.5	3.7	8.1	84
10	3.9	4.4	10.7	111
33	3.7	3.5	8.1	84
100	3.4	3.6	7.7	80
333 (1)	(2)	-	-	-

Solvent control = dimethyl sulfoxide

(1) [redacted] precipitated in the exposure medium

(2) Cell death

(3) $\text{Total growth} = \text{Cell count after 24 h treatment} \times \frac{\text{Cell count after 24 h subculture}}{\text{Cells subcultured after treatment (1.6 x } 10^5 \text{ c/ml)}}$

TABLE 3 CYTOTOXIC AND MUTAGENIC RESPONSE OF [redacted] IN THE MOUSE LYMPHOMA L5178Y TEST SYSTEM

EXPERIMENT 1

Dose (µg/ml)	Cell count after treatment % of controls	CE after treatment % of controls	Actual survival of the cells % of controls[1]	CE at day 3 absolute %	Total no. of wells with mutants Total (small large)	Mutation frequency x 10^5 Total (small large)
		Without metabolic activation				
		3 hours treatment				
SC1	100	100	100	105	17 (3s 14l)	1.7 (0.3s 1.4l)
SC2	--	--	--	89	12 (2s 10l)	1.4 (0.2s 1.2l)
20	101	90	91	118	14 (3s 11l)	1.3 (0.3s 1.0l)
30	101	97	98	125	11 (3s 8l)	0.9 (0.2s 0.7l)
40	91	63	57	115	16 (4s 12l)	1.5 (0.4s 1.1l)
50	89	100	89	107	27 (2s 25l)	2.7 (0.2s 2.5l)
60	64	100	64	88	22 (4s 18l)	2.7 (0.5s 2.2l)
70	76	85	65	95	16 (1s 15l)	1.8 (0.1s 1.7l)
80	48	98	47	97	27 (3s 24l)	3.0 (0.3s 2.7l)
90	24	90	22	95	37 (8s 29l)	4.3 (0.9s 3.4l)
EMS	93	116	108	115	144 (10s 134l)	16.3 (1.1s 15.2l)
		With 8% (v/v) metabolic activation				
		3 hours treatment				
SC1	100	100	100	85	11 (5s 6l)	1.4 (0.6s 0.8l)
SC2	--	--	--	108	12 (4s 8l)	1.2 (0.4s 0.8l)
10	89	93	83	90	10 (2s 8l)	1.2 (0.2s 1.0l)
50	106	91	96	107	14 (7s 7l)	1.4 (0.7s 0.7l)
100	108	60	65	84	6 (3s 3l)	0.7 (0.4s 0.4l)
130	87	97	84	115	7 (2s 5l)	0.6 (0.2s 0.4l)
175	82	112	92	95	12 (4s 8l)	1.3 (0.4s 0.9l)
230	75	93	70	115	14 (2s 12l)	1.3 (0.2s 1.1l)
275	59	90	53	147	14 (2s 12l)	1.0 (0.1s 0.9l)
300	63	55	35	105	26 (8s 18l)	2.7 (0.8s 1.9l)
DMN	100	87	87	64	90 (18s 72l)	16.7 (3.3s 13.4l)

CE = Cloning Efficiency; EMS = Ethylmethanesulphonate; DMN = Dimethylnitrosamine; s = small colonies; l = large colonies
SC = Solvent control = dimethyl sulfoxide

(1) The actual survival of cells =the cell count after treatment x the CE after treatment
(% of controls) (% of controls) (% of controls)

TABLE 4 CYTOTOXIC AND MUTAGENIC RESPONSE OF [redacted]B IN THE MOUSE LYMPHOMA L5178Y TEST SYSTEM

EXPERIMENT 2

Dose (µg/ml)	Cell count after treatment % of controls	CE after treatment % of controls	Actual survival of the cells % of controls[2]	CE at day 3 absolute %	Total no. of wells with mutants Total (small large)	Mutation frequency x 10^5 Total (small large)
		Without metabolic activation				
		24 hours treatment				
SC1	100	100	100	97	22 (2s 20l)	2.4 (0.2s 2.2l)
SC2	--	--	--	104	21 (3s 18l)	2.2 (0.3s 1.9l)
10	97	86	83	89	10 (1s 9l)	1.2 (0.1s 1.1l)
50	113	97	110	76	26 (6s 20l)	3.7 (0.9s 2.8l)
75	129	44	57	65	26 (4s 22l)	4.3 (0.7s 3.6l)
100	99	84	83	85	21 (3s 18l)	2.6 (0.4s 2.2l)
130	67	65	44	86	33 (11s 22l)	4.2 (1.4s 2.8l)
175	44	81	36	84	23 (9s 14l)	2.9 (1.1s 1.8l)
200	20	73	15	84	29 (4s 25l)	3.7 (0.5s 3.2l)
225	12	63	8	79	27 (8s 19l)	3.7 (1.1s 2.6l)
EMS	111	44	49	67	301 (33s 268l)	91.5 (10.0s 81.5l)
		With 12% (v/v) metabolic activation				
		3 hours treatment				
SC1	100	100	100	98	14 (1s 13l)	1.5 (0.1s 1.4l)
SC2	--	--	--	107	21 (4s 17l)	2.1 (0.4s 1.7l)
130	98	104	102	118	20 (1s 19l)	1.8 (0.1s 1.7l)
175	105	99	104	121	16 (4s 12l)	1.4 (0.4s 1.1l)
225	108	88	95	107	18 (1s 17l)	1.8 (0.1s 1.7l)
250	99	104	103	93	14 (3s 11l)	1.6 (0.3s 1.3l)
275	118	71	84	110	25 (5s 20l)	2.4 (0.5s 1.9l)
300	79	94	74	85	21 (3s 18l)	2.6 (0.4s 2.2l)
325 [1]	64	125	80	108	14 (1s 13l)	1.4 (0.1s 1.3l)
350 [1]	78	97	76	80	16 (3s 13l)	2.1 (0.4s 1.7l)
DMN	99	89	88	74	112 (29s 83l)	18.6 (4.8s 13.8l)

CE = Cloning Efficiency; EMS = Ethylmethanesulphonate; DMN = Dimethylnitrosamine; s = small colonies; l = large colonies
SC = Solvent control = dimethyl sulfoxide
(1) [redacted]precipitated in the exposure medium
(2) The actual survival of cells =the cell count after treatment x the CE after treatment
(% of controls) (% of controls) (% of controls)

APPENDIX 1

INDIVIDUAL COLONY COUNTS AND CELL COUNTS DURING EXPRESSION PERIOD

- Cell counts during expression period
- Cloning efficiency immediately after exposure
- Mutation experiments, individual colony counts

Tables 5-8 Experiment 1
Tables 9-12 Experiment 2

Abbreviations used: DMN, dimethylnitrosamine
EMS, ethylmethanesulphonate
Solvent control, SC dimethyl sulfoxide

APPENDIX 1 continued

EXPERIMENT 1

TABLE 5 CELL COUNTS AND SUBCULTURE DATA
Without metabolic activation

	DAY 0				DAY 2			DAY 3	
Dose (µg/ml)	Total amount of cells before treatment x 10^6	Total amount of cells after treatment x 10^6	% (1)	Subculture x 10^6 total amount(3)	Cell count c/ml x 10^5	% (2)	Subculture x 10^6 total amount(4)	Cell count c/ml x 10^5	%(2)
SC1	8	5.1	64	4.0	4.8	100	4.0	6.4	100
SC2	8	4.6	58	4.0	3.9	--	4.0	6.2	--
0.5 (5)	8	5.5	69	4.0	5.7	131	4.0	5.1	81
1 (5)	8	5.5	69	4.0	5.1	117	4.0	5.2	83
5 (5)	8	5.0	63	4.0	4.7	108	4.0	6.1	97
10 (5)	8	5.7	71	4.0	4.7	108	4.0	5.5	87
20	8	4.9	61	4.0	5.2	120	4.0	4.6	73
30	8	4.9	61	4.0	4.6	106	4.0	5.1	81
40	8	4.4	55	4.0	3.8	87	4.0	6.0	95
50	8	4.3	54	4.0	3.8	87	4.0	5.9	94
60	16	6.2	39	5.9	4.0	92	4.0	5.9	94
70	16	7.4	46	7.1	3.7	85	4.0	5.7	90
80	16	4.7	29	4.6	3.0	69	4.0	6.0	95
90	16	2.3	14	2.3	2.5	57	4.0	4.6	73
EMS	8	4.5	56	4.0	3.9	90	4.0	6.0	95

(1) $\frac{\text{cells after treatment}}{\text{cells before treatment}} \times 100\%$

(2) $\frac{\text{cell count}}{\text{cell count of \{SC1+SC2\}/2}} \times 100\%$

(3) cell density 0.4 x 10^5 c/ml

(4) cell density 1.6 x 10^5 c/ml

(5) not used for the mutation assay

APPENDIX 1 continued

EXPERIMENT 1

TABLE 6 CELL COUNTS AND SUBCULTURE DATA
With metabolic activation (8% (v/v) S9 fraction)

	DAY 0				DAY 2			DAY 3	
Dose (µg/ml)	Total amount of cells before treatment x 10^6	Total amount of cells after treatment x 10^6	% (1)	Subculture x 10^6 total amount(3)	Cell count c/ml x 10^5	% (2)	Subculture x 10^6 total amount(4)	Cell count c/ml x 10^5	% (2)
SC1	8	5.2	65	4.0	5.2	100	4.0	6.3	100
SC2	8	5.4	68	4.0	4.8	--	4.0	5.4	--
1 (5)	8	5.3	66	4.0	4.2	84	4.0	5.7	97
10	8	4.7	59	4.0	3.5	70	4.0	6.8	116
30 (5)	8	4.3	54	4.0	4.3	86	4.0	5.6	96
50	8	5.6	70	4.0	3.8	76	4.0	6.2	106
70 (5)	8	4.8	60	4.0	4.4	88	4.0	5.9	101
100	8	5.7	71	4.0	4.7	94	4.0	6.0	103
130	8	4.6	58	4.0	4.8	96	4.0	6.0	103
175	16	8.7	54	8.0	5.4	108	4.0	6.0	103
230	16	7.9	49	7.6	4.2	84	4.0	5.7	97
275	16	6.3	39	5.9	3.0	60	4.0	6.0	103
300	16	6.7	42	6.4	2.2	44	4.0	6.1	104
DMN	8	5.3	66	4.0	2.1	42	4.0	4.3	74

(1) $\frac{\text{cells after treatment}}{\text{cells before treatment}} \times 100\%$

(2) $\frac{\text{cell count}}{\text{cell count of \{SC1+SC2\}/2}} \times 100\%$

(3) cell density 0.4 x 10^5 c/ml
(4) cell density 1.6 x 10^5 c/ml
(5) not used for the mutation assay

APPENDIX 1 continued

EXPERIMENT 1

TABLE 7 CLONING EFFICIENCY DAY 0

Dose (µg/ml)	Empty wells/ cloning plate 1	2	Total no. of empty wells	Cloning efficiency absolute	relative (% of control) (1)
			Without metabolic activation		
SC1	29	31	60	116	100
SC2	38	38	76	93	-
0.5	28	37	65	108	103
1	36	37	73	97	93
5	37	40	77	91	87
10	31	35	66	107	102
20	38	37	75	94	90
30	33	37	70	101	97
40	53	46	99	66	63
50	36	31	67	105	100
60	29	38	67	105	100
70	44	35	79	89	85
80	30	39	69	102	98
90	36	39	75	94	90
EMS	27	30	57	121	116
			With metabolic activation (8% (v/v) S9 fraction)		
SC1	28	22	50	135	100
SC2	32	30	62	113	-
1	36	29	65	108	87
10	30	31	61	115	93
30	41	43	84	83	67
50	28	34	62	113	91
70	36	37	73	97	78
100	43	49	92	74	60
130	23	35	58	120	97
175	20	28	48	139	112
230	34	27	61	115	93
275	27	36	63	111	90
300	48	49	97	68	55
DMN	29	36	65	108	87

(1) $\frac{\text{Cloning efficiency absolute}}{\{\text{Cloning efficiency SC1+SC2}\}/2} \times 100\%$

APPENDIX 1 continued

EXPERIMENT 1

TABLE 8 SELECTION DATA AND CLONING EFFICIENCY

Dose (µg/ml)	Mutant colonies: Number of wells with mutants per selection plate								Mutant colonies: Total number of mutants			Cloning efficiency (at day 3): No. of empty wells per cloning plate		Cloning efficiency (at day 3): Total number of empty wells	Cloning efficiency (at day 3): CE x 100%	Mutation frequency per 10^5 survivors		
	1		2		3		4					1	2			total	small	large
Without metabolic activation																		
	s	l	s	l	s	l	s	l	s	l	s+l							
SC1	0	3	0	3	2	5	1	3	3	14	17	32	35	67	105	1.7	0.3	1.4
SC2	1	2	0	3	0	1	1	4	2	10	12	40	39	79	89	1.4	0.2	1.2
20	1	4	0	1	0	3	2	3	3	11	14	29	30	59	118	1.3	0.3	1.0
30	2	2	1	1	0	2	0	3	3	8	11	29	26	55	125	0.9	0.2	0.7
40	2	3	0	4	1	3	1	2	4	12	16	30	31	61	115	1.5	0.4	1.1
50	1	4	0	6	0	5	1	10	2	25	27	38	28	66	107	2.7	0.2	2.5
60	1	2	1	6	1	6	1	4	4	18	22	38	42	80	88	2.7	0.5	2.2
70	0	3	0	5	1	4	0	3	1	15	16	35	39	74	95	1.8	0.1	1.7
80	0	7	1	8	1	6	1	3	3	24	27	35	38	73	97	3.0	0.3	2.7
90	3	9	2	8	1	6	2	6	8	29	37	36	38	74	95	4.3	0.9	3.4
EMS	3	35	2	36	2	33	3	30	10	134	144	36	25	61	115	16.3	1.1	15.2
With metabolic activation (8% (v/v) S9 fraction)																		
	s	l	s	l	s	l	s	l	s	l	s+l							
SC1	1	4	4	1	0	1	0	0	5	6	11	41	41	82	85	1.4	0.6	0.8
SC2	1	3	0	3	1	1	2	1	4	8	12	35	30	65	108	1.2	0.4	0.8
10	1	3	0	1	1	3	0	1	2	8	10	41	37	78	90	1.2	0.2	1.0
50	2	3	1	1	1	2	3	1	7	7	14	35	31	66	107	1.4	0.7	0.7
100	0	0	1	1	1	2	1	0	3	3	6	40	43	83	84	0.7	0.4	0.4
130	0	2	1	1	0	1	1	1	2	5	7	31	30	61	115	0.6	0.2	0.4
175	0	1	1	2	2	1	1	4	4	8	12	34	40	74	95	1.3	0.4	0.9
230	0	2	1	3	1	3	0	4	2	12	14	29	32	61	115	1.3	0.2	1.1
275	1	3	0	3	0	4	1	2	2	12	14	21	23	44	147	1.0	0.1	0.9
300	1	4	2	6	1	2	4	6	8	18	26	26	41	67	105	2.7	0.8	1.9
DMN	4	18	5	16	2	19	7	19	18	72	90	49	52	101	64	16.7	3.3	13.4

s = small colonies
l = large colonies

APPENDIX 1 continued

EXPERIMENT 2

TABLE 9 CELL COUNTS AND SUBCULTURE DATA
Without metabolic activation

	DAY 0	DAY 1			DAY 3	
Dose (µg/ml)	Total amount of cells before treatment x 10^6	Total amount of cells after treatment x 10^6	% (1)	Subculture X 10^6 Total amount(3)	Cell count c/ml x 10^5	%(2)
SC1	8	5.2	65	4.0	4.4	100
SC2	8	4.9	61	4.0	4.1	--
10	8	4.9	61	4.0	4.9	115
50	8	5.7	71	4.0	4.9	115
75	8	6.5	81	4.0	4.6	108
100	8	5.0	63	4.0	4.2	99
130	8	3.4	43	3.0	4.2	99
175	8	2.2	28	2.0	4.8	113
200	8	1.0	13	0.9	4.3	101
225	8	0.6	8	0.5	5.3	125
250 (5)	16	(4)	-	-	-	-
275 (5)	16	(4)	-	-	-	-
300 (5)	16	(4)	-	-	-	-
325 (5,6)	16	(4)	-	-	-	-
EMS	8	5.6	70	4.0	2.9	68

(1) $\frac{\text{cells after treatment}}{\text{cells before treatment}} \times 100\%$

(2) $\frac{\text{cell count}}{\text{cell count of \{SC1+SC2\}/2}} \times 100\%$

(3) cell density 0.4 x 10^5 c/ml
(4) cell death after treatment
(5) not used for the mutation assay
(6) [redacted] precipitated in the exposure medium

APPENDIX 1 continued

EXPERIMENT 2

TABLE 10 CELL COUNTS AND SUBCULTURE DATA
With metabolic activation (12% (v/v) S9 fraction)

	DAY 0				DAY 2			DAY 3	
Dose (µg/ml)	Total amount of cells before treatment x 10^6	Total amount of cells after treatment x 10^6	% (1)	Subculture x 10^6 total amount(3)	Cell count c/ml x 10^5	% (2)	Subculture x 10^6 total amount(4)	Cell count c/ml x 10^5	%(2)
SC1	8	7.2	90	4.0	5.2	100	4.0	6.1	100
SC2	8	6.7	84	4.0	5.4	--	4.0	6.0	--
10 (5)	8	6.7	84	4.0	4.9	92	4.0	5.5	91
50 (5)	8	7.6	95	4.0	4.8	91	4.0	5.9	98
100 (5)	8	6.4	80	4.0	5.7	108	4.0	5.3	88
130	8	6.8	85	4.0	5.3	100	4.0	5.3	88
175	8	7.3	91	4.0	5.0	94	4.0	4.9	81
225	8	7.5	94	4.0	4.6	87	4.0	6.2	102
250	8	6.9	86	4.0	5.2	98	4.0	5.5	91
275	8	8.2	103	4.0	4.6	87	4.0	5.1	84
300	16	11.0	69	8.0	5.1	96	4.0	5.3	88
325 (6)	16	8.9	56	8.0	5.3	100	4.0	5.3	88
350 (6)	16	10.8	68	8.0	4.2	79	4.0	6.1	101
DMN	8	6.9	86	4.0	3.1	58	4.0	3.4	56

(1) $\frac{\text{cells after treatment}}{\text{cells before treatment}} \times 100\%$

(2) $\frac{\text{cell count}}{\text{cell count of \{SC1+SC2\}/2}} \times 100\%$

(3) cell density 0.4 x 10^5 c/ml

(4) cell density 1.6 x 10^5 c/ml

(5) not used for the mutation assay

(6) [redacted] precipitated in the exposure medium

APPENDIX 1 continued

EXPERIMENT 2

TABLE 11 CLONING EFFICIENCY DAY 0

Dose (µg/ml)	Empty wells/ cloning plate 1	2	Total no. of empty wells	Cloning efficiency absolute	relative (% of control) (1)
			Without metabolic activation		
SC1	47	36	83	84	100
SC2	57	47	104	61	-
10	54	49	103	62	86
50	46	49	95	70	97
75	69	71	140	32	44
100	51	53	104	61	84
130	54	66	120	47	65
175	56	50	106	59	81
200	57	56	113	53	73
225	57	64	121	46	63
250	(3)				
275	(3)				
300	(3)				
325 (2)	(3)				
EMS	68	71	139	32	44
			With metabolic activation (12% (v/v) S9 fraction)		
SC1	36	34	70	101	100
SC2	32	39	71	99	-
10	31	33	64	110	110
50	31	46	77	91	91
100	30	31	61	115	115
130	34	34	68	104	104
175	36	35	71	99	99
225	33	47	80	88	88
250	30	38	68	104	104
275	47	47	94	71	71
300	38	37	75	94	94
325 (2)	28	27	55	125	125
350 (2)	41	32	73	97	97
DMN	38	41	79	89	89

(1) Cloning efficiency absolute ___ x 100%
{Cloning efficiency SC1+SC2}/2

(2) [redacted] precipitated in the exposure medium

(3) Cell death after treatment

APPENDIX 1 continued

EXPERIMENT 2

TABLE 12 SELECTION DATA AND CLONING EFFICIENCY

Dose (µg/ml)	Mutant colonies: Number of wells with mutants per selection plate								Mutant colonies: Total number of mutants			Cloning efficiency (at day 3): No. of empty wells per cloning plate		Cloning efficiency (at day 3): Total number of empty wells	Cloning efficiency (at day 3): CE x 100%	Mutation frequency per 10^5 survivors		
	1		2		3		4					1	2			total	small	large
Without metabolic activation																		
	s	l	s	l	s	l	s	l	s	l	s+l							
SC1	0	4	2	4	0	7	0	5	2	20	22	36	37	73	97	2.4	0.2	2.2
SC2	0	3	2	4	1	6	0	5	3	18	21	39	29	68	104	2.2	0.3	1.9
10	1	2	0	3	0	1	0	3	1	9	10	42	37	79	89	1.2	0.1	1.1
50	2	5	2	5	2	7	0	3	6	20	26	42	48	90	76	3.7	0.9	2.8
75	0	7	1	4	1	5	2	6	4	22	26	46	54	100	65	4.3	0.7	3.6
100	0	4	1	3	2	7	0	4	3	18	21	44	38	82	85	2.6	0.4	2.2
130	0	8	4	4	6	8	1	2	11	22	33	37	44	81	86	4.2	1.4	2.8
175	4	2	1	4	2	2	2	6	9	14	23	40	43	83	84	2.9	1.1	1.8
200	0	8	2	7	0	6	2	4	4	25	29	41	42	83	84	3.7	0.5	3.2
225	2	6	2	3	2	5	2	5	8	19	27	41	46	87	79	3.7	1.1	2.6
EMS	8	69	5	65	10	66	10	68	33	268	301	48	50	98	67	91.5	10.0	81.5
With metabolic activation (12% (v/v) S9 fraction)																		
	s	l	s	l	s	l	s	l	s	l	s+l							
SC1	0	6	0	3	0	1	1	3	1	13	14	34	38	72	98	1.5	0.1	1.4
SC2	2	3	1	7	1	2	0	5	4	17	21	33	33	66	107	2.1	0.4	1.7
130	1	5	0	3	0	7	0	4	1	19	20	34	25	59	118	1.8	0.1	1.7
175	1	4	1	5	0	1	2	2	4	12	16	24	33	57	121	1.4	0.4	1.1
225	0	4	0	5	1	5	0	3	1	17	18	33	33	66	107	1.8	0.1	1.7
250	1	3	1	5	0	0	1	3	3	11	14	32	44	76	93	1.6	0.3	1.3
275	1	6	1	5	1	5	2	4	5	20	25	33	31	64	110	2.4	0.5	1.9
300	0	3	0	3	3	5	0	7	3	18	21	34	48	82	85	2.6	0.4	2.2
325 (1)	0	3	0	2	1	3	0	5	1	13	14	39	26	65	108	1.4	0.1	1.3
350 (1)	0	3	2	7	1	0	0	3	3	13	16	42	44	86	80	2.1	0.4	1.7
DMN	4	23	9	17	8	25	8	18	29	83	112	43	49	92	74	18.6	4.8	13.8

s = small colonies

l = large colonies

(1) [redacted] precipitated in the exposure medium

APPENDIX 2

CERTIFICATE OF ANALYSIS

[redacted]

Certificate of Analysis [redacted]

[redacted]
page 1 of 2

ICS-331

Product name : [redacted]
Chemical name : [redacted]
Batch number : [redacted]

Test results:

Method	Analysis of	Unit	Result *1
Jo/72.11, Jo/95.2	Peroxidic compounds (sum) ***See page 2 for a specification***	% m/m	28.6 (± 1.5)
J20010792	[redacted]		
J20010792	[redacted]	% m/m	2.0 (± 0.3)
Amp/88.9	Water	% m/m	2.6 (± 0.3)
J20010792	Unidentified impurities	% m/m	0.5 (± 0.2)

*1 bracketed values are estimated 95% confidence intervals

File code : [redacted]
Analytical documentation : 20010792

[redacted]

APPENDIX 2 continued

[redacted]

Certificate of Analysis [redacted]

[redacted]

[redacted] batch 1510-14: specification of the peroxidic compounds

structure	% m/m
[redacted]	

[redacted]